

Capillarization of Hepatic Sinusoid by Liver Endothelial Cell-Reactive Autoantibodies in Patients with Cirrhosis and Chronic Hepatitis

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The special features of liver sinusoidal endothelium (LSE) are crucial for normal liver physiology. Cirrhotic livers, especially in primary biliary cirrhosis (PBC), are characterized by transformation of the LSE into a continuous, vascular type. The transformation is important for disease progression and explains some of the pathological hallmarks of the cirrhotic liver. Here, we investigated the presence of liver sinusoidal endothelial cell (LSEC)-reactive autoantibodies (Abs) in the sera of patients with autoimmune liver diseases, and assessed the ability of these Abs to transform LSE into vascular endothelium. Compared to healthy individuals (9%), significantly higher numbers of patients with PBC (59%; $P < 0.001$) and autoimmune hepatitis (AIH) (32%; $P < 0.05$) had Abs against LSECs. Incubation of primary LSEC cultures with F(ab')₂ fragments of anti-LSEC Abs isolated from sera of patients with PBC and AIH, induced 1) cell surface expression of vascular endothelium-associated markers, CD31, and factor VIII-related antigen; 2) significant production of fibronectin, laminin and collagen type IV; 3) loss of fenestrae, formation of tight junctions and Weibel-Palade bodies. Deposition of immunoglobulins on LSECs were found in liver biopsies of AIH and PBC patients. Thus, anti-LSEC autoAbs transform LSE into a vascular type and may therefore play an important role in the development of hepatocellular failure and portal hypertension in PBC and AIH patients. (*Am J Pathol* 2003, 163:1275–1289)

Liver sinusoidal endothelial cells (LSECs) differ morphologically and functionally from capillary endothelial cells of other organs. They possess typical fenestrations clustered in sieve plates.¹ Discontinuous sinusoidal endothe-

lial cells differ also phenotypically from vascular or continuous endothelial cells, for instance in their failure to express factor VIII-related antigen (FVIIIIRAg), platelet-endothelial cell adhesion molecule 1 (PECAM-1 or CD31), CD34, and E-selectin.² They have no basement membrane and only an attenuated extracellular matrix (ECM), consisting mostly of fibronectin (FN).¹ Capillarization of LSECs is well described and common to cirrhosis. In chronic hepatitis and cirrhosis, LSECs frequently undergo transformation to a vascular type with the formation of a true basement membrane.^{3,4} Morphological transformation of LSECs to vascular-type endothelial cells in patients with primary biliary cirrhosis (PBC) has been reported,⁵ while another study suggested endothelial cell damage in PBC and to a lesser extent in other liver diseases.⁶ The unique arrangement of the normal sinusoidal endothelium is likely to facilitate the large exchanges that take place between hepatocytes and the blood. It is known that the formation of basement membrane and changes in LSECs will interfere with the bi-directional exchange of molecules and therefore have deleterious effects on liver physiology, such as decreased sinusoidal compliance with increased resistance to blood flow, and may contribute to development of portal hypertension in PBC. Babbs et al⁵ have discussed other consequences that may result from these changes, such as development of cirrhosis by causing ischemic atrophy of hepatocytes, thereby leading to increased fibrogenesis and compensatory hypertrophy of surrounding hepatocytes. All these changes may result in the development of hepatocellular failure. Thus, morphological transformation of LSECs to vascular-type endothelial cells in patients with PBC and autoimmune hepatitis (AIH) may have important clinical consequences.

AIH, PBC, and primary sclerosing cholangitis (PSC) are regarded as autoimmune liver diseases (ALDs).⁷ AIH

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and PBC are inflammatory liver diseases, in which hepatocytes⁸ and small bile ducts⁹ are destroyed, respectively. PSC, while considered by many to be an autoimmune hepatobiliary disease, has multiple features that differ from not only classical autoimmunity but also from both AIH and PBC. PSC is characterized by a destruction of both extra- and intrahepatic bile ducts, leading to strictures and dilatations.¹⁰ AIH and PBC present with a 90% female predominance, which is typical for autoimmune diseases in general. In contrast, PSC is characterized by a 60% male predominance.

Both organ- and non-organ specific autoantibodies (Abs) are detected in ALDs.⁷ Abs commonly found in all three groups of patients are smooth muscle cell antibodies (SMA) and anti-nuclear antibodies (ANA).⁷ Some AIH patients are further characterized by the presence of antibodies to liver-kidney microsomal fractions (LKM) and soluble liver antigens (SLA).^{11,12} PBC patients are diagnosed by the presence of anti-mitochondrial antibodies (AMA),¹³ while PSC patients are characterized by the presence of a perinuclear cytoplasmic immunofluorescent staining of neutrophils (p-ANCA).¹⁴ In most cases these Abs are directed to intracellular antigens and have not been shown to be associated with any clinical parameter. However, they remain good diagnostic markers for these diseases.

In the present study, we were interested in detecting the occurrence of Abs to cell-surface-expressed molecules on liver sinusoidal endothelial cells (LSECs) in sera of ALD patients for two reasons: 1) endothelial cells are the "gatekeepers" of organs/tissues from the perspective of the recipient's blood stream. It is likely that the endothelium of an organ suffers the major and the first insult by the recipient's immune system as they form the first line of contact with the circulating immune cells and antibodies; and 2) The reports of sinusoidal endothelial cell transformation³⁻⁵ and damage⁶ in patients with chronic liver injury indicated that these cells may be targets of immune attack. Thus it seemed reasonable to question whether Abs to LSECs occur in the sera of ALD patients and may contribute to the pathogenesis of these diseases.

We therefore investigated the presence of Abs to surface antigens expressed on LSECs in ALD patients and the clinical relevance was determined by testing the ability of these Abs to transform LSECs to a vascular type. The reversibility of the transformation process was also determined *in vitro* by removal of these Abs.

Materials and Methods

Patients

Sera from normal healthy individuals ($n = 33$) and patients with various stages of PBC ($n = 27$, 3 males), AIH ($n = 34$, 9 males), and PSC ($n = 47$, 37 males) were tested for presence and functional capacity of autoantibodies that reacted with unstimulated and cytokine stimulated LSECs. The diagnosis of AIH, PBC, and PSC were based on accepted histological, clinical, and cholangiographic criteria. The diagnosis of AIH was based on the

diagnostic criteria presented by the International AIH group.¹⁵ All AIH patients had a score >15 before steroid treatment. PBC diagnosis was based on the presence of AMA, cholestatic liver test abnormalities, and a liver biopsy consistent with PBS. All PSC patients had ERCP/MRCP changes characteristic for sclerosing cholangitis. Secondary causes to sclerosing cholangitis were carefully excluded in all patients. The majority had involvement of both the intra- and extrahepatic biliary tree, but 25% had only intrahepatic involvement. Moreover, a liver biopsy consistent with PSC was present in all patients. None of the patients had an ongoing infection, were pregnant, or had received blood transfusions during the last 6 months before investigation. All were negative for hepatitis B and C infections.

The average age for AIH, PBC, and PSC patients was 51, 60, and 53 years, respectively. In patients who had undergone liver transplantation all blood samples tested were obtained before the operation. In PSC patients with an associated inflammatory bowel disease the colonic disease was in remission as judged by clinical and endoscopic criteria. Twenty of the PSC patients were treated with ursodeoxycholic acid. All of the AIH patients were treated with steroids and/or azathioprin, while all of the PBC patients were treated with ursodeoxycholic acid. Seven of 34 AIH (21%), 11 of 27 PBC (41%), and 25 of 47 PSC (53%) patients had end-stage disease, ie, were accepted for liver transplantation due to signs of liver failure. Sera from 30 patients with viral hepatitis, two with alcoholic steatohepatitis, three with non-alcoholic steatohepatitis, five with rheumatoid arthritis, five with systemic lupus erythematosus, and five with Wegener's granulomatosis were also included as controls.

Isolation of Sinusoidal Endothelial Cells

Human LSECs were isolated from the liver of one normal healthy liver donor using a method similar to one already described before.¹⁶ In short, all identifiable vascular structures were excised from the liver specimen. Liver tissue was mechanically disrupted (into small cubes) and enzymatically digested with dispase (1.6 units/ml) overnight at 4°C. The individual cubes were then mechanically disaggregated with a flat instrument to release the endothelial cells. The decanted cell suspension was centrifuged at $666 \times g$ at 4°C for 10 minutes. LSECs were then isolated on a density gradient of 35% percoll (Sigma, St. Louis, MO) at $5000 \times g$ at 4°C for 10 minutes. The top band of the gradient was collected and the cells were washed in PBS. The isolated cells were seeded on gelatin-coated tissue-culture flasks and grown at 37°C in a humidified atmosphere of 5% CO₂ in air. Cell monolayers were passaged at confluence using trypsin-EDTA. LSECs were routinely cultured in an endothelial cell selective medium MCDB 131 (Invitrogen, Carlsbad, CA) containing 10% heat-inactivated human AB serum. The medium was further supplemented with endothelial cell growth medium (EGM-2) singlequots obtained from Cambrex Bio Science Walkersville (Clonetics, Walkersville, MD). Permission for the present study was granted from the local ethical committee.

Table 1. Phenotypic Comparison of Isolated Liver Sinusoidal Endothelial Cells with Human Umbilical Vein Endothelial Cells

Surface marker	LSECs	HUVECs
CD31	—	++
FVIIIIRAg	—	++
CD34	—	+
E-selectin	—	++*
Cadherin-5	—	+
VCAM-1	++*	++*
ICAM-1	++	++
CD4	+	—
CD14	+	—
CD32	++	—
Wheat germ agglutinin	+++	++

*Detected only after activation with TNF- α and IFN- γ —, negative; +, positive; ++, strongly positive.

Human kidney microvascular endothelial cells (HKMECs) were isolated as described earlier (¹⁷). Human umbilical vein endothelial cells (HUVECs) were purchased from Clonetics.

Phenotyping of LSECs

Single-color fluorescence was used to phenotypically characterize LSECs. Primary antibodies for staining were fluorescein isothiocyanate (FITC)-conjugated antibodies to anti-CD4, -CD14, -CD31, -CD32, -CD34, -cadherin-5, and FVIIIIRAg, as well as phycoerythrin (PE)- conjugated anti-VCAM or -E-selectin (Biogenesis, Poole, Dorset, UK) antibodies and non-conjugated anti-fibroblast and smooth muscle cell (α -actin) antibodies (Harlan Sera-

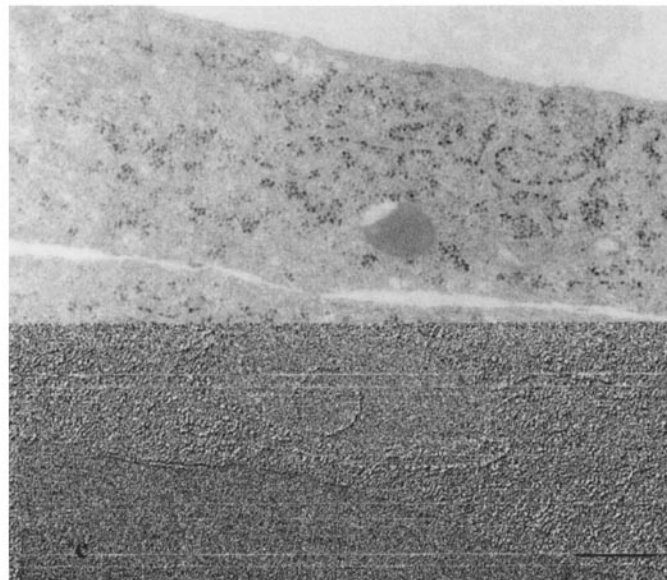
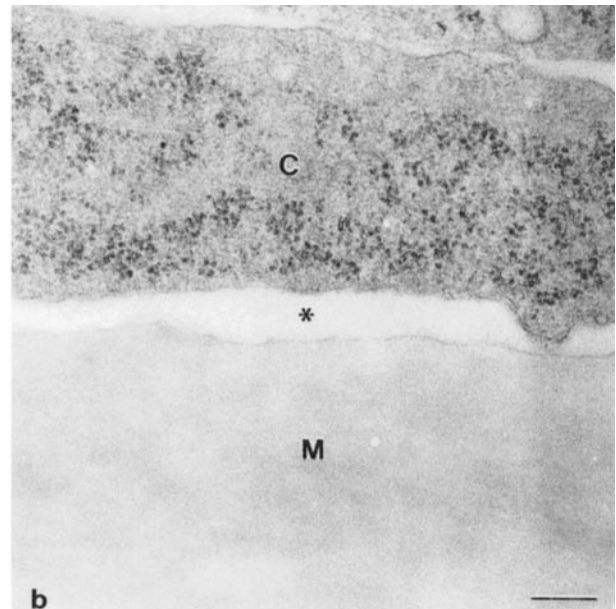
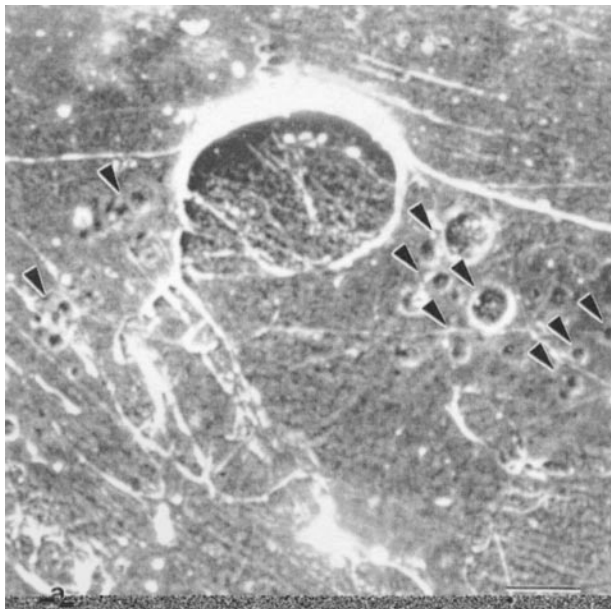


Figure 1. **a:** Scanning electron micrograph showing liver endothelial cells cultured in transwell tissue culture inserts displaying fenestrations (arrowheads) in the cytoplasm. **b:** TEM micrograph showing an endothelial cell (C) attached to the membrane (M) without any matrix (*). **c:** No formation of tight junctions are seen. Bars: **a**, 1 μ m; **b**, 200 nm; **c**, 1 μ m.

Table 2. Binding of Autoantibodies in Sera of Patients with Autoimmune Diseases to Sinusoidal Endothelial Cells

Patient group	Total no. of sera with positive reactivity to LSECs/titer	Positive with unstimulated + stimulated* LSECs	Positive with only stimulated LSECs	Positive with stimulated HUVECs	Positive with stimulated HKMECs
PBC (<i>n</i> = 27)	16 (59%)/1:10→1:50	14	2	3	3
AIH (<i>n</i> = 34)	11 (32%)/1:10→1:50	11	0	5	4
PSC (<i>n</i> = 47)	5 (11%)/1:5–1:10	5	0	3	2
Hepatitis C (<i>n</i> = 22)	1 (4%) 1:5	0	1	1	1
Hepatitis B (<i>n</i> = 8)	1 (12%) 1:5	0	1	1	1
Steatohepatitis (<i>n</i> = 5)	0	0	0	0	0
RA (<i>n</i> = 5)	0	0	0	4	0
SLE (<i>n</i> = 5)	0	0	0	5	1
WG (<i>n</i> = 5)	0	0	0	2	4
Normals (<i>n</i> = 33)	3 (9%)/<1:5	3	0	3	3

*. LSECs were stimulated overnight with IFN- γ + TNF- δ .

RA, rheumatoid arthritis; SLE, systemic lupus erythematosus; WG, wegeners granulomatosis. (PBC vs. norm and AIH vs. norm; *P* = 0.0001 and *P* < 0.05, respectively).

Lab, Loughborough, Leicestershire, UK). FITC-conjugated goat anti-mouse secondary antibodies (Jackson ImmunoResearch, West Grove, PA) were also used. Intracellular staining for the CD31 and FVIIIIRAg was also performed using 5% saponin-treated LSECs.

Scanning Electron Microscopy

LSECs were grown to confluency on membrane filters in 24-well plates. The membranes were prepared for SEM as described elsewhere.¹⁸ In short, membranes were fixed in 2% glutaraldehyde, briefly rinsed in distilled water, placed in 70% ethanol for 10 minutes, and in 99.5% ethanol for 15 minutes, all at 4°C, and dried. After drying, the membranes were cut off, mounted on an aluminum stub, and coated with 15 nm platinum (Polaron Components Group, Watford, Herts, UK). The samples were analyzed in a Jeol JSM-820 scanning electron microscope at 15 kV.

Transmission Electron Microscopy (TEM)

Cells were fixed as described above and further procedure was followed as described elsewhere.¹⁸ In short, after fixation, the membrane was cut free, fixed for 1 hour

at 4°C in a buffer containing 0.15 mol/L sodium cacodylate, 1% osmium tetroxide, and 3 mmol/L CaCl₂, pH 7.4. Subsequently, the wells were rinsed briefly, in 0.15 mol/L sodium cacodylate buffer, dehydrated in ethanol as described above, and imbedded in Spurr resin (Agar Scientific Ltd., Essex, UK). The sections were contrasted with uranyl acetate followed by lead citrate, and examined at 80 kV in a Leo 906 (Oberkochen, Germany) transmission electron microscope.

Determination of the Presence of Anti-LSEC Reactive Antibodies in Sera of ALD Patients

For the flow cytometric assay, unstimulated/stimulated LSECs/HKMECS/HUVECs were used and the procedure carried out as described earlier.¹⁷ Briefly, 5×10^5 cells were incubated with 50 μ L of patient serum for 1 hour at 22°C, and then washed three times with PBS. Ten μ L of 1:4 diluted fluoresceinated F(ab')₂ fragments of goat anti-human IgG (Fc-specific) and anti-human IgM (μ -chain specific) (Jackson ImmunoResearch) antibodies were added and incubated at 4°C on ice in the dark for 25 minutes. The cells were washed and then analyzed on a Becton Dickinson flow cytometer (FACSorter; BD Biosciences, San Jose, CA). Fluorescence signals from

Table 3. Cytokine Production (pg/ml) in Supernatants of LSECs Incubated with Anti-LSEC Antibodies

Cytokine	PBC F(ab) ₂ Ig frac. 12 hours	PBC F(ab) ₂ Ig frac. 72 hours	AIH F(ab) ₂ Ig frac. 12 hours	AIH F(ab) ₂ Ig frac. 72 hours	Norm. F(ab) ₂ Ig frac. 12 hours	Norm. F(ab) ₂ Ig frac. 72 hours
IL-1 β	<30	<30	<30	<30	<30	<30
IL-2	<30	<30	<30	<30	<30	<30
IL-4	<30	<30	<30	<30	<30	<30
IL-5	<30	<30	<30	<30	<30	<30
IL-6	450 \pm 80	4680 \pm 125	500 \pm 90	4900 \pm 132	400 \pm 30	6120 \pm 113
IL-8	62500 \pm 132	243100 \pm 128	69600 \pm 143	264000 \pm 147	3100 \pm 90	4050 \pm 90
IL-12	<30	<30	<30	<30	<30	<30
TGF- β	900 \pm 25	840 \pm 45	850 \pm 33	835 \pm 23	2000 \pm 124	2100 \pm 120
TNF- α	<30	<30	<30	<30	<30	<30
IFN- γ	<30	<30	<30	<30	<30	<30
MCP-1	283 \pm 30	1448 \pm 48	287 \pm 23	1745 \pm 35	70 \pm 20	90 \pm 30

\pm = Standard deviation.
frac., fraction; Norm., normal.

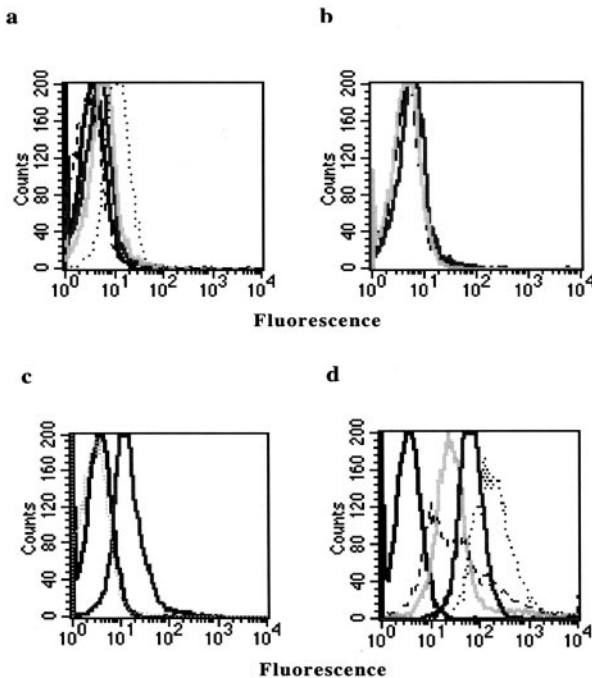


Figure 2. **a:** Normal LSECs did not express CD31 (dark gray line), FVIIIIRAg (light gray line), and VCAM (dashed line) but expressed ICAM (dotted line). **b:** Treatment of LSECs with cytokines TNF- α and IFN- γ did not induce the cell surface expression of these molecules. **c:** CD31 (dark gray line) but not FVIIIIRAg (light gray line) was expressed intracellularly in normal saponin-treated LSECs. **d:** Treatment of LSECs with anti-LSEC Abs from PBC and AIH patients induced the expression of CD31 (dark gray line), FVIIIIRAg (light gray line), and VCAM (dashed line), and increased expression of ICAM (dotted line). Secondary antibodies served as negative control (black line). A representative histogram from one patient is shown.

10,000 cells were counted and the percentage of FITC-positive cells was recorded. A shift in the mean fluorescence of 20 channels in the test sample as compared to negative control was considered as positive, determined as described before.¹⁷ All sera giving a positive reaction were further diluted (1:5, 1:10, 1:100) in PBS to determine the titer of the antibodies.

In all experiments, one set of cell samples remained untreated, while another set was stimulated with recombinant TNF- α and IFN- γ (20 ng/ml and 200 ng/ml, respectively; R&D systems, Abingdon, UK) which was added to the culture medium overnight before harvesting of cells for analysis.

Purification of IgG Antibodies from Sera of ALD Patients with Anti-LSEC Antibodies, and Preparation of F(ab')₂ Fragments

IgG fractions were isolated from the sera of eight AIH and 10 PBC patients with LSEC antibodies using goat anti-human IgG agarose beads (Sigma) according to standard procedure. IgG fractions were also purified from five normal controls.

For isolation of anti-LSEC IgG F(ab')₂ fragments from PBC and AIH patients, an ImmunoPure F(ab')₂ preparation kit was used according to the instructions of the manufacturer (Pierce Biotechnology, Rockford, IL). The purity of antibody and antibody fragment preparations were verified by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) following silver staining of the gels. The purified IgG F(ab')₂ fragments from eight AIH, 10 PBC, and five normals were concentrated individually and each fraction was used at a concentration of 3 mg/ml for the following experiments.

Cytokine Production by LSECs Cultures Treated with Anti-LSEC Antibodies

We were interested in investigating whether anti-LSEC Abs could induce production of various inflammatory cytokines. For this purpose, purified PBC, AIH, and normal IgG F(ab')₂ fractions diluted in medium as stated above were added to LSECs (1×10^6 cells) and the culture supernatants were collected after 12 and 72 hours, sterile-filtered, and kept frozen at -70°C until as-

Table 4. Induction of Expression of Vascular Endothelial Cell-Associated and Various Immune Recognition Molecules on LSECs by Treatment with Anti-LSEC Antibodies for 12 and 72 Hours

Antibodies to	Unstimulated LSECs	TNF- α and IFN- γ stimulated LSECs	LSECs + PBC F(ab') ₂ Ig fractions	LSECs + AIH F(ab') ₂ Ig fractions	LSECs + norm F(ab') ₂ Ig fractions
CD31	—	—	+ (12 hrs) ++ (72 hrs)	+ (12 hrs) ++ (72 hrs)	—
FVIIIIRAg	—	—	+ (12 hrs) ++ (72 hrs)	+ (12 hrs) ++ (72 hrs)	—
HLA-DR	—	+++*	—	—	—
CD80	—	—	+	—	—
CD44	+++	+++	+++	+++	+++
CD58	+	+	+	+	+
CD40	—	+	+	+	+
ICAM-1	++	+++	++/+++	++/+++	+
VCAM-1	—	++	+ (12 hrs) ++ (72 hrs)	+ (12 hrs) ++ (72 hrs)	—
E-selectin	—	—	—	—	—
Cadherin-5	—	—	—	—	—
CD34	—	—	—	—	—

*Only after 3 days with IFN- γ .

+, >10–25; ++, >26–50; +++, >51–75 mean fluorescence channels as compared to negative control (only secondary antibodies)

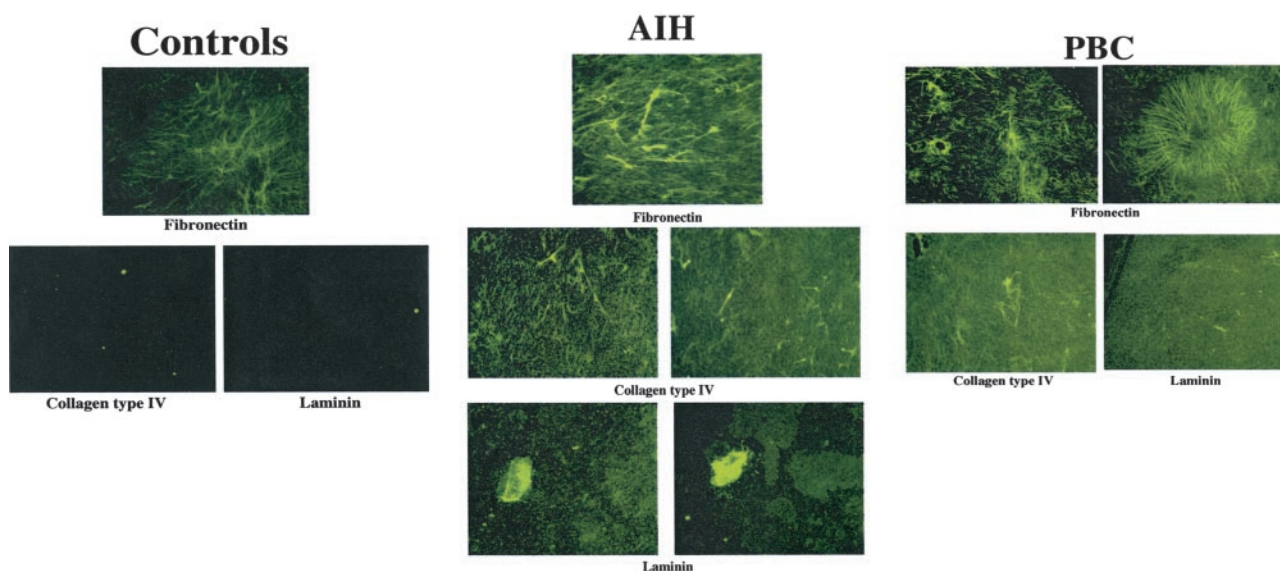


Figure 3. a: Normal liver sinusoidal endothelial cells did not produce the basement membrane components collagen type IV or laminin, nor did treatment with immunoglobulin fractions from normal control sera induce their production. However, some deposition of fibronectin was observed. **b** and **c:** Intense staining for the deposition of fibronectin, collagen type IV, and laminin was observed by LSECs after treatment for 72 hours with anti-LSEC Abs from PBC and AIH patients, respectively. A representative result from each patient group is shown.

sayed. The cytokines and chemokines (Table 3) were measured by standard sandwich ELISA techniques using Quantikine sandwich enzyme immunoassay from R&D Systems (Minneapolis, MN). Assays were performed according to the manufacturer's instructions.

Pretreatment with Anti-LSEC Antibodies to Detect Activation of LSECs

We investigated whether the IgG F(ab')₂ fractions from AIH and PBC could induce vascular endothelial cell-associated markers on LSECs as well as the expression of immune recognition elements, known to facilitate leukocyte-endothelial cell interactions. Experiments were performed as described earlier.¹⁹ Monoclonal antibodies to various cell surface markers (Table 4) were used. Isotype control antibodies were also used. After incubation on ice at 4°C the cells were washed and analyzed by flow cytometry.

Detection of Basement Membrane Production by LSECs Treated with Anti-LSEC Autoantibodies

1×10^6 LSECs were seeded in four six-well tissue culture plates. One plate was left untreated, while the others were incubated with normal/PBC/AIH IgG F(ab')₂ fractions. After 72 hours of incubation, cells were detached using PBS containing 1 mmol/L EDTA.

Immunocytochemistry

The wells (after detachment of cells) were stained for 1 hour at 4°C, with the following antibodies, rabbit anti-laminin (Roche Molecular Biochemicals, Mannheim, Ger-

many), working dilution 1:100, rabbit anti-type IV collagen (Dakopatts, Glostrup, Denmark), working dilution 1:100, and mouse anti-fibronectin (Harlan Sera-Lab), working dilution 1:25. Wells were washed three times with PBS and stained with FITC-conjugated goat-anti-rabbit and goat anti-mouse secondary antibodies, respectively, for 1 hour at 4°C. Plates were visualized under a fluorescence microscope.

Western Blot Analysis

The detached cells (1×10^6) were also tested for basement membrane formation using the standard SDS-PAGE and Western blot analysis. The same primary antibodies as stated above were used and the secondary antibodies were horseradish peroxidase-conjugated F(ab')₂ fragments of goat anti-rabbit and goat anti-mouse secondary antibodies. Purified proteins of laminin, fibronectin, and collagen type IV served as controls. Antibody-binding components were detected using the ECL kit (Amersham Biosciences Inc., Piscataway, NJ). Chemiluminescence was detected using a Fluor-S Max (Bio-Rad, Hercules, CA) equipped with a CCD camera operating at -35°C.

Detection for Basement Membrane Production by LSECs Treated with IgG F(ab')₂ Fractions Known to Contain Anti-Mitochondrial or Anti-Nuclear Antibodies but Not Anti-LSEC Antibodies

For this purpose, 200 μ l of PBC and AIH IgG F(ab')₂ fractions were absorbed with 5×10^6 packed LSECs at 4°C for 24 hours. The cells were centrifuged and the supernatant transferred to a new tube with freshly packed

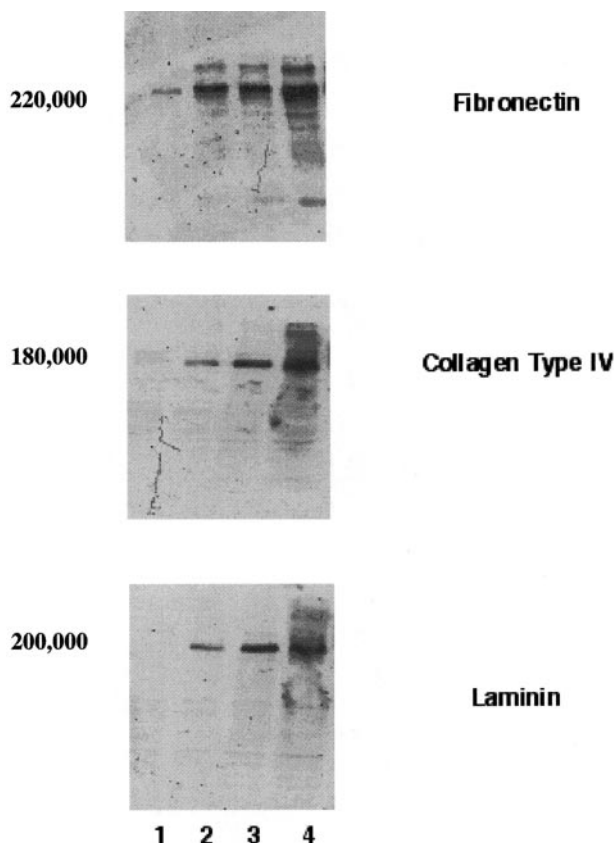


Figure 4. Western blot analysis of cell lysates from 1×10^6 untreated LSECs (lane 1), LSECs treated with IgG F(ab')₂ fractions from PBC patients (lane 2), LSECs treated with IgG F(ab')₂ fractions from AIH patients (lane 3), and protein controls (lane 4) stained with anti-fibronectin, anti-collagen type IV, and anti-laminin antibodies. Controls consisted of recombinant human fibronectin, collagen type IV, and laminin (lane 4). Under reducing conditions fibronectin was detected as a Mr 220,000 band, collagen type IV as Mr 180,000 band and laminin as a Mr 200,000 band. Normal LSECs expressed small amounts of fibronectin but not collagen type IV and laminin. However, anti-LSEC Abs induced expression of all three basement membrane proteins. A representative result from each patient group is shown.

LSECs. This procedure was performed two more times. The absorbed IgG F(ab')₂ fractions were retested with LSECs using flow cytometry to detect efficiency of absorption. The unabsorbed and the successfully absorbed IgG F(ab')₂ fractions were tested for the presence of AMA and ANA using the ELISA assay. Routinely performed immunohistochemical staining on rat liver/kidney tissue and Hep-2 cells was used. Further detection for the presence of M2 mitochondrial antigen using the commercially available ELISA kit (Medical and Biological Lab. Cat. Nagoya, Japan) was done and the procedure followed as described by the manufacturers.

Reverse Transcriptase-Polymerase Chain Reaction

RNA Preparation and Reverse Transcription

RNA was extracted from approximately 10^6 untreated normal LSECs, LSECs treated with IgG F(ab')₂ fragments from AIH and PBC sera and from HUVECs (control) according to the instructions of the Qiagen RNA Blood

Minikit (Qiagen, Hilden, Germany). RNA was eluted with 30 μ l of RNase free water.

The cDNA synthesis was performed in a total volume of 50 μ l containing 30 μ l RNA, 1X first-strand buffer (50 mmol/L Tris-HCl pH 8.3, 75 mmol/L KCl, 3 mmol/L MgCl₂), 105 μ g/ml pdN₆ (Amersham Biosciences Inc., Uppsala, Sweden), 1 mmol/L of each dNTP (Amersham Biosciences), 1 mmol/L dithiothreitol, 0.48 units/ μ l RNasin (Promega, Madison, WI) and 4.8 units/ μ l M-MLV RT (Invitrogen, Paisley, Scotland, UK), at 37°C for 1.5 hours. The reaction was stopped by heating at 68°C for 15 minutes.

PCR

5 μ l of cDNA was used in a 25- μ l PCR-reaction containing 1X PCR buffer (10 mmol/L Tris-HCl, pH 8.3, 50 mmol/L KCl, 1.5 mmol/L MgCl₂, 0.001% gelatin), 200 μ mol/L of each dNTP (Applied Biosystems/Roche, Branchburg, NJ), 5% glycerol (Sigma), 100 ng/ μ l cresol red (Sigma), 0.03 units/ μ l AmpliTaq polymerase (Applied Biosystems) and 0.5 μ mol/L of each primer. Primer sequences were: ABL-F: 5'-CGG CTC TCG GAG GAG ACG ATG A-3'; ABL-R: 5'-CCC AAC CTT TTC GTT GCA CTG T-3'; factor VIIIIRAg-F: 5'-CCA GGG ACC TTG GAG ATA-3'; factor VIIIIRAg-R: 5'-AAG ACG CTC TGG GCG AGG AAC-3'; CD31-F: 5'-CCA CTG CAG AGT ACC AGG TGT TGG-3'; CD31-R: 5'-ATC GAG AAG GAG CGT TTC T-3'.

PCR conditions were 94°C for 4 minutes followed by 32 PCR amplification cycles. The first 10 cycles were done in a two-segment step at 94°C for 30 seconds and at 61°C for 1 minute. The next 22 cycles were done in a three-segment step at 94°C for 15 seconds, 59°C for 50 seconds, and 72°C for 30 seconds. Five μ l of the PCR products were run in a ready-to-use PAGE system (Amersham Biosciences). 12.5% non-denaturing polyacrylamide gels were run for 1.5 hours and visualized after an automated silver staining procedure (Amersham Biosciences).

Real-Time PCR

2.5 μ l of cDNA was used in a 25 μ l of reaction containing 1X SYBR (Applied Biosystems, Foster City, CA) and 300 nmol/L of each primer. Primer sequences for CD31 and FVIIIIRAg was the same as above while Abelson (ABL) primers were: ABL-RT-F: 5'-CGA AGG GAG GGT GTA CCA TTA C-3' and ABL-RT-R: 5'-CGT TGA ATG ATG AAC CAA CTC-3'. The PCR reaction was performed and analyzed on the ABI 7000 Sequence Detection System with the following PCR conditions: 50°C for 2 minutes, 95°C for 10 minutes, followed by 40 PCR amplification cycles with 95°C for 15 seconds and 58°C for 1 minute. Relative quantification of gene expression was calculated according to the Delta-Delta Ct method (Applied Biosystems; user bulletin 2). ABL was used as an endogenous reference and unstimulated LSEC was used as calibrator.

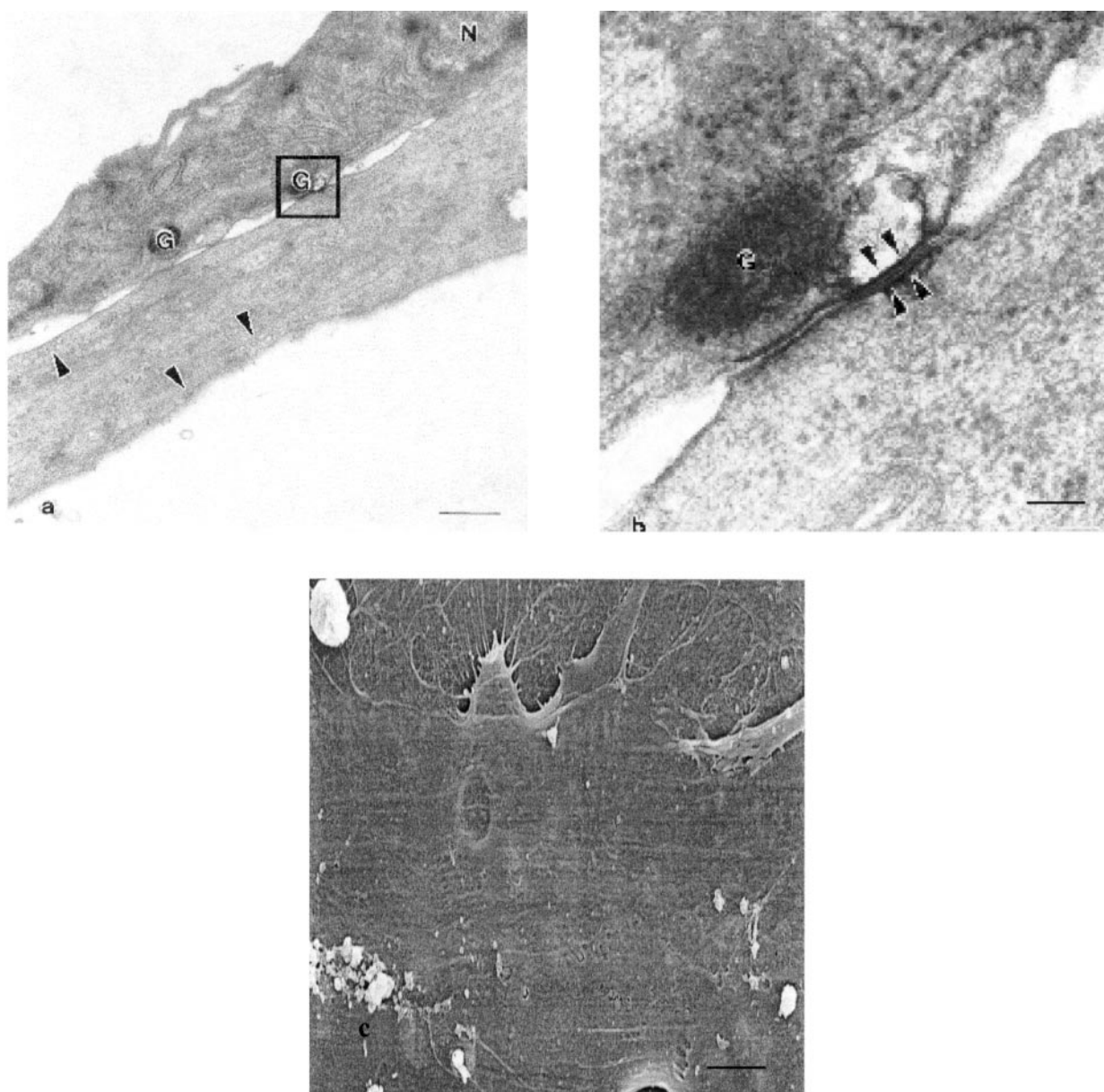


Figure 5. TEM and SEM micrographs of transformed cultured LSECs after treatment with anti-LSECs from PBC and AIH patients. **a:** TEM micrograph showing adjacent endothelial cells containing endothelial-specific granulae (G) (Weibel-Palade bodies) and pinocytotic vesicles (arrowheads). N, nucleus. **b:** Enlargement from the area marked as a **box** showing a tight junction (arrowheads) between two endothelial cells. G, Weibel-Palade bodies. **c:** SEM micrograph showing loss of fenestrae. Bars: **a**, 1 μ m; **b**, 100 nm; **c**, 1 μ m.

Reversibility of Vascular Transformation to a Sinusoid

We checked whether transformed LSECs could revert to a sinusoid. For this purpose, vascular transformation was induced as described earlier, using anti-LSEC F(ab')₂ fragments from four PBC and four AIH sera (Table 6). No patients with end-stage diseases were included for this analysis. The F(ab')₂ fragments were removed from culture after 72 hours and the medium was changed every day for 1 week to remove the unbound (by the process of shedding) antibodies in the supernatant. No positive binding of anti-IgG and IgM to cultured LSECs was observed after 1 week, indicating the complete absence of

bound antibodies to LSECs. Cells were cultured with only growth medium and analyzed at various time intervals, eg, 1, 2, 3, 4, and 8 weeks. Cells were detached at each time-point and analyzed for surface expression of CD31, FVIIIIRAg, basement membrane production, and detection of fenestrae using electron microscopy.

Liver Biopsy Staining

Liver biopsies were obtained from five PBC, five AIH, three PSC patients and three normal liver transplant donors. Hepatectomy specimens were snap frozen in liquid nitrogen and stored at -70°C . 5- μ m cryostat sections

Table 5. Effects of PBC and AIH F(ab)₂ IgG Fractions Before and After Absorption with Liver Sinusoidal Endothelial Cells

Action	PBC		AIH	
	Before absorption	After absorption	Before absorption	After absorption
Abs to mitochondrial antigens	++(1:400)	++(1:380)	—	—
Abs to smooth muscle cells	+	+	+	+
Abs to HUVECs	++	++	++	++
Abs to HKMECs	+	+	+	+
Abs to LSECs	+++	—	+++	—
Induction of laminin/fibronectin/ collagen type IV	+++	—	+++	—
Induction of CD31	++	—	++	—
Induction of FVIIIIRAg	++	—	++	—

were fixed in acetone at room temperature, and immunohistochemistry was performed by double immunostaining, using FITC-conjugated monoclonal antibodies against DC-SIGN2, also called liver/lymph node-specific ICAM-3-grabbing non-integrin (L-SIGN) (R&D systems), an antibody specific for LSECs, and cyanine-3-conjugated F(ab')₂ fragments of goat anti-human IgG (Fc-specific) or IgM antibodies (Jackson ImmunoResearch). After preliminary blocking with normal bovine serum, sections were incubated with antibodies for 1 hour at room temperature, washed, and counterstained with Mayer's hematoxylin (Sigma). Sections were analyzed with a laser-scanning confocal microscopy MCR-500 system, equipped with a krypton-argon laser, for coincident sites of reactivity.

Statistical Analysis

Mann-Whitney *U*-test was used to compare quantitative variables between two groups and Kruskal-Wallis test was used when comparisons between more than two groups were done. χ^2 test and, when appropriate, Fisher's exact test were used to compare categorical parameters. Differences were considered significant if $P < 0.05$.

Results

Phenotypic and Electron Microscopic Analysis of the Isolated Liver Sinusoidal Endothelial Cells

No cell surface expression of CD31, FVIIIIRAg, CD34, E-selectin, or cadherin-5 on either unstimulated or cytokine-stimulated LSECs was observed (Table 1). The cells, however, expressed CD4, CD14, CD32 (Fc γ receptor II), and VCAM (after activation with cytokine). Scanning and transmission electron microscopic analysis of LSECs revealed the presence of fenestrae and absence of a basement membrane. No presence of Weibel-Palade bodies or formation of tight junctions was observed, confirming the sinusoidal nature of the isolated endothelial cells (Figure 1).

Significantly Higher Numbers of PBC and AIH Patients Have Abs to Liver Sinusoidal Endothelial Cells

In all, a significantly higher fraction of PBC (16 of 27; 59%; $P = 0.0001$) and AIH (11 of 34; 32%; $P < 0.05$) but not PSC (5 of 47; 11%; $P =$ not significant) patients, had Abs to LSECs as compared to normal controls (3 of 33; 9%) (Table 2). PBC *versus* AIH ($P < 0.05$), PBC *versus* PSC ($P = 0.0001$), and PSC *versus* AIH ($P =$ not significant). In general, a lower frequency of anti-HUVEC and anti-HKMEC Abs as compared to anti-LSEC Abs was observed in ALD patients (Table 2).

Immunoglobulin Class and Titers of Anti-LSEC Abs in Sera of ALD Patients

In general, patients with PBC, AIH had a mixture of anti-LSEC Abs that belonged to both IgM and IgG classes. In PBC, two sera had only IgM, four only IgG, and six had a mixture of IgG+M immunoglobulins, while in AIH, two had only IgG and nine had a mixture of IgG+M. In PSC and normals mainly IgM antibodies were detected. In addition, higher titers of anti-LSEC Abs were detected in PBC and AIH patients as compared to controls (Table 2).

Detection of IL-8 and MCP-1 in LSEC Culture Supernatants Containing Anti-LSEC Abs from PBC and AIH Patients

Anti-LSECs F(ab')₂ from PBC and AIH patients induced LSECs to produce significantly higher levels of IL-8 and MCP-1 at 12 hours and 72 hours compared to F(ab')₂ from normals ($P < 0.001$) (Table 3). Antibody-negative sera from the same patient groups did not induce the production of these chemokines. Interestingly, the production of TGF- β 1 and IL-6 was higher in LSEC supernatants following incubation with F(ab')₂ fractions of nor-

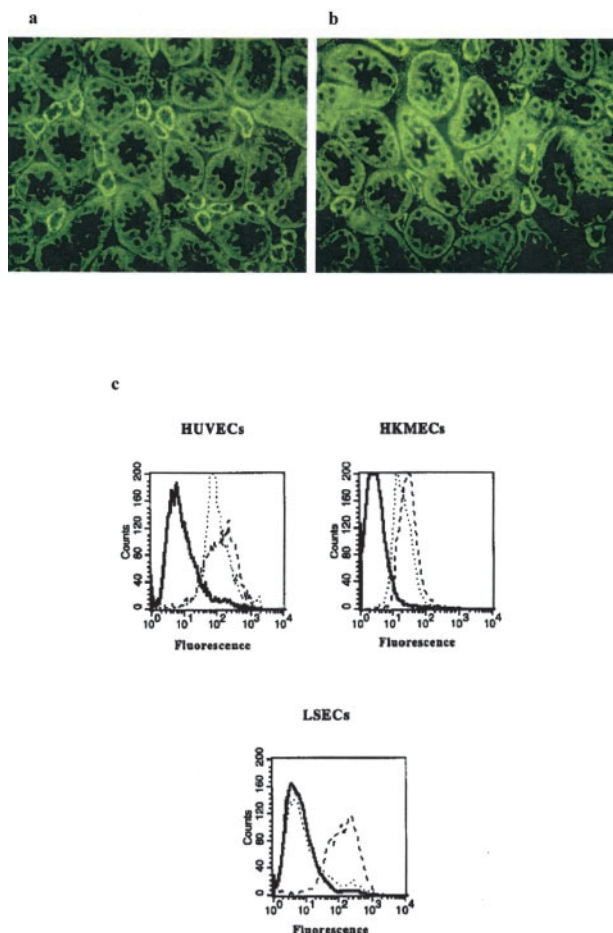


Figure 6. Staining of rat kidney tissue for anti-mitochondrial antibodies showed the presence of these antibodies before (a) and even after (b) absorption with LSECs of immunoglobulin fractions from PBC patients. c: A representative histogram of flow cytometric analysis from one PBC patient showing the presence of reactivity to HUVECs and HKMECs before (dashed lines) and after (dotted lines) absorption with LSECs. However, all reactivity to LSECs was completely removed after absorption. Similar results were observed with AIH patients.

mal controls as compared to those induced by the IgG F(ab')₂ fractions in PBC and AIH patients.

Anti-LSEC Abs from PBC and AIH Patients Induce Expression of CD31, FVIIIIRAg, and VCAM, and Increased Expression of ICAM-1 on LSECs

Neither normal (Figure 2a; Table 4) nor cytokine-stimulated (Figure 2b; Table 4) LSECs expressed CD31 or FVIIIIRAg on the cell surface. Interestingly, normal LSECs intracellularly expressed CD31 but not FVIIIIRAg (Figure 2c). However, IgG F(ab')₂ fractions from PBC and AIH patients induced the expression of CD31, FVIIIIRAg, and VCAM and increased expression of ICAM on LSECs, while normal IgG F(ab')₂ fractions did not (Figure 2d; Table 4). The expression of other molecules was not altered by treatment for 12 hours with PBC, AIH, or normal IgG F(ab')₂ fractions (Table 4).

Production of Basement Membrane Induced by Anti-LSEC Abs from PBC and AIH Patients

No production of the basement membrane components laminin and collagen type IV was detected in normal LSECs. However, some deposition of fibronectin was detected (Figure 3). LSECs treated for 72 hours with anti-LSEC reactive IgG F(ab')₂ fractions from PBC and AIH patients showed intense production of collagen type IV, laminin, and fibronectin (Figure 3). Western blot analysis of untreated LSECs and LSECs treated with IgG F(ab')₂ fractions from PBC and AIH patients gave similar results (Figure 4). In addition, transmission and scanning electron microscopy revealed the presence of Weibel-Palade bodies, formation of tight junctions and decreased fenestrae (Figure 5). LSECs treated with normal controls did not show deposition of any of the basement membrane components tested.

Evidence that Basement Membrane Production is Caused by Anti-LSEC Abs and Not AMA, ANA, anti-HUVEC, or HKMEC Abs

When retested, binding of anti-LSEC Abs to LSECs was completely abolished after absorption with LSECs, confirming efficiency of absorption. AMA and ANA were detected in both the unabsorbed and absorbed IgG F(ab')₂ fractions from PBC patients. No significant change in the titer of these Abs was observed after absorption with LSECs (Table 5; Figure 6). Similarly, absorbed and unabsorbed IgG F(ab')₂ fractions from AIH patients showed presence of ANA Abs. In addition, the absorbed IgG F(ab')₂ fractions retained reactivity to HUVEC and HKMECs, indicating the presence of heterogeneous anti-endothelial cell specificities. Treatment of LSECs with absorbed IgG F(ab')₂ fractions did not induce the production of laminin, collagen type IV, or the expression of CD31 and FVIIIIRAg.

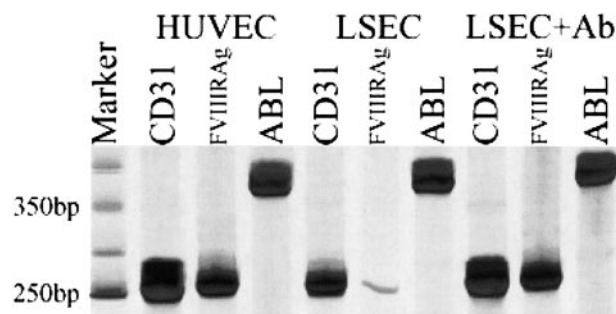


Figure 7. Gene expression of CD31, FVIIIIRAg, and ABL in HUVECs, unstimulated LSECs and LSEC treated with the F(ab')₂ fragments of anti-LSEC Abs from PBC/AIH patients (LSEC+Ab). ABL is an endogenous reference gene used for correction of variations in cell number. Abs-treated LSEC had higher gene expression of CD31 and FVIIIIRAg as compared to unstimulated LSEC. Quantitative analysis with real-time RT-PCR showed that the expression of CD31 and vWF had increased 3 log and 4.5 log, respectively.

Table 6. Effect on Liver Sinusoidal Endothelial Cells at 8 Weeks of Addition and Subsequent Removal after 72 Hours of 3 mg/ml IgG F(ab)₂ Fractions from Patients with Autoimmune Hepatitis and Primary Biliary Cirrhosis

AIH pts titers of anti-LSEC Abs	Exp. of CD31 Abs+	Exp. of CD31 Abs-	Exp. of FVIIIIRAg Abs+	Exp. of FVIIIIRAg Abs-	Prod. of fibronectin Abs+	Prod. of fibronectin Abs-	Prod. of collagen type IV Abs+	Prod. of collagen type IV Abs-	Prod. of laminin Abs+	Prod. of laminin Abs-	Pr. of fenestrae Abs+	Pr. of fenestrae Abs-
1 (1:5)	++	-	++	-	+++	+	+++	-	+++	-	-	+++
2 (1:10)	++	-	++	-	+++	+	+++	-	+++	-	-	+++
3 (1:20)	++	-	++	-	+++	+	+++	-	+++	-	-	++
4 (1:50)	++	-	++	-	+++	+	+++	-	+++	-	-	+
PBC pts. titres of Abs												
1 (1:5)	++	-	++	-	+++	+	+++	+	+++	+	-	-
2 (1:10)	++	+/++	++	+/++	+++	+++	+++	+++	+++	+++	-	-
3 (1:20)	++	+/++	++	+/++	+++	+++	+++	+++	+++	+++	-	-
4 (1:50)	++	+/++	++	+/++	+++	+++	+++	+++	+++	+++	-	-

Abs+, liver sinusoidal endothelial cells treated with antibodies; Abs-, liver sinusoidal endothelial cells after removal of antibodies; -, negative; +, weak; ++, moderate; +++, strong expression; Exp., expression; Prod., production; Pr., presence.

LSECs Expressed mRNA for CD31 and FVIIIIRAg

Even though normal LSECs did not express CD31 and FVIIIIRAg on the cell surface, mRNAs for both of these molecules were present in these cells (Figure 7). Abs-treated LSECs had higher gene expression of CD31 and FVIIIIRAg as compared to normal untreated LSECs. Quantitative analysis with real-time PCR showed that the expression of CD31 and FVIIIIRAg had increased 3 log and 4.5 log, respectively.

In Vitro Reversibility of the Transformation Process

As soon as 1 week after removal of anti-LSEC Abs, transformed LSECs partially reverted to a sinusoid, with loss of CD31 and FVIIIIRAg expression and decreased basement membrane production. By 4 weeks the transformed LSECs had completely reverted to sinusoid. This phenomenon was observed in all cases of LSECs treated with AIH but only in one case treated with PBC F(ab')₂ fractions (Table 6). Electron microscopy showed formation of fenestrae in 4 of 4 AIH-treated LSECs but not in those treated with PBC samples. Monitoring of LSECs with PBC samples for 3 months did not show reversibility of transformation.

Deposition of Immunoglobulins in the Livers of PBC and AIH Patients that Bind to Sinusoidal Endothelial Cells

Figure 8a shows the staining pattern of LSECs in normal, PBC, and AIH livers using the antibody DC-SIGN2. Weak binding of immunoglobulins to LSECs in normal and PSC livers was observed; however, intense binding of IgG and IgM to LSECs in PBC and AIH patients livers was seen as detected by the staining pattern of CY3-conjugated anti-IgG and IgM antibodies (Figure 8b). Double staining with both antibodies showed that in many areas coincident sites of reactivity (yellow) was observed (Figure 8c). Interestingly, in general, PBC liver sections showed a

dense or more intense deposition of immunoglobulins around the sinusoidal endothelial cells as compared to AIH specimens, where a finer deposition was observed (see Figure 8c).

Clinical Correlation of Presence of Anti-LSEC Antibodies

Among the 11 PBC patients with end-stage disease, eight (73%) had these antibodies and among those 16 patients without end-stage disease 50% had LSEC autoantibodies (*P* = not significant). In the AIH group seven patients had end-stage disease and 43% of them had autoantibodies to LSEC compared to 30% in the group without end-stage disease (*P* = not significant).

Discussion

In the present study we demonstrated that a significantly higher percentage of PBC and AIH patients, as compared to PSC and normal individuals, have autoantibodies to surface antigens expressed on liver sinusoidal endothelial cells. In addition, we have shown that these Abs are capable of transforming LSECs into a vascular type. The clinical relevance of the anti-LSEC Abs is supported by the *in vivo* deposition of IgG and IgM immunoglobulins on the sinusoidal endothelial cells in biopsy sections of PBC and AIH livers. Thus, for the first time we report the presence and functional capacity of Abs to surface antigens expressed on liver sinusoidal endothelial cells in PBC and AIH patients. The binding of F(ab')₂ fragments of anti-LSECs Abs may result in the production of a signal(s) that transforms the endothelial phenotype from that of a sinusoid into a vascular one, and consequently play an important role in the pathogenesis and progression of PBC and AIH. It is important to mention that patients with viral hepatitis and non-alcoholic and alcoholic-steatohepatitis did not demonstrate the presence of these antibodies.

In chronic hepatitis and cirrhosis, LSECs frequently undergo transformation into capillary or vascular endothelial cells possessing a basement membrane and no

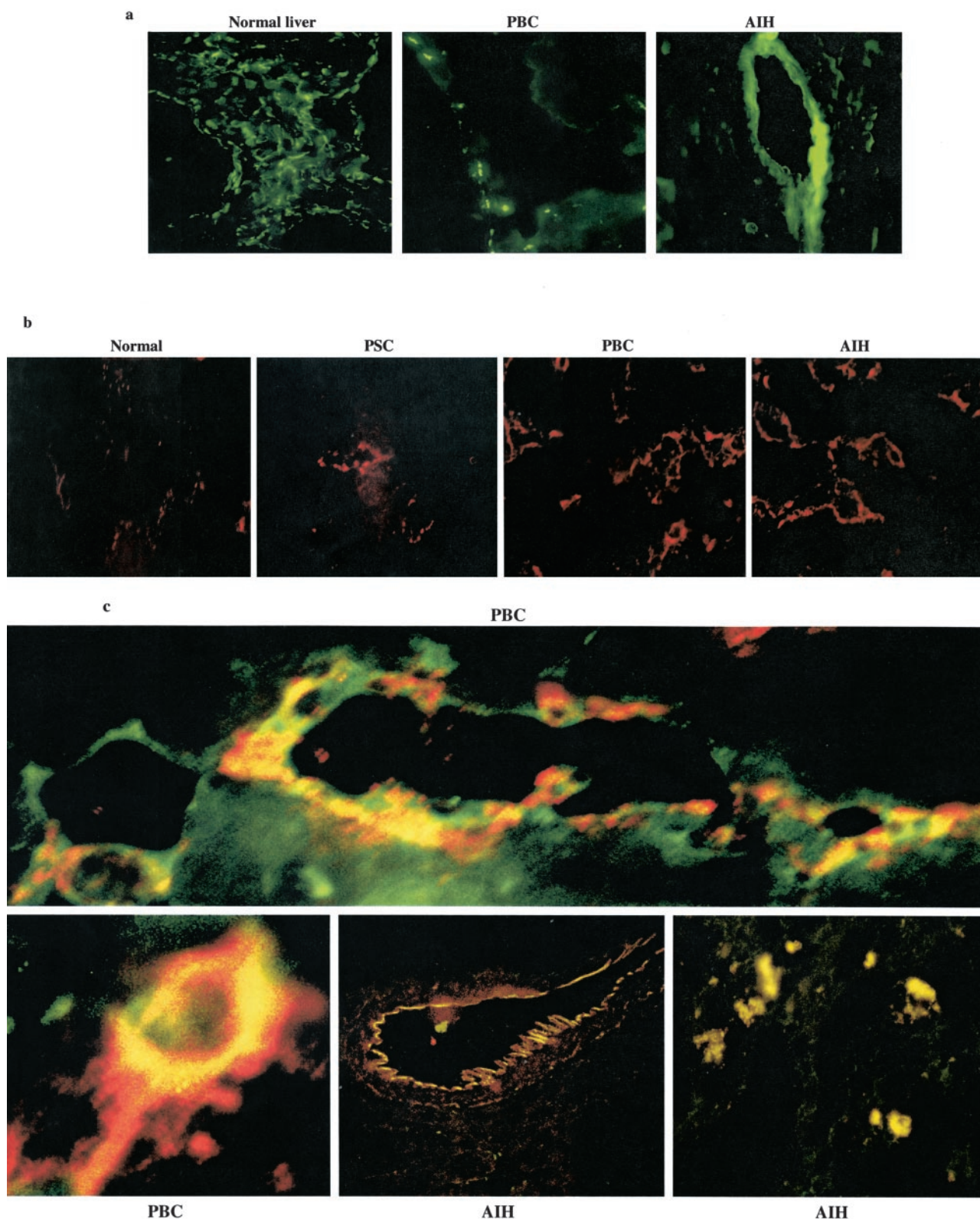


Figure 8. a: Staining pattern of sinusoidal endothelial cells in normal, PBC, and AIH liver sections. Sections were stained with anti-DC-SIGN2, an antibody specific for sinusoidal endothelial cells of the liver. **b:** Staining liver sections with anti-human IgG and IgM showed weak or almost no deposition of immunoglobulins in the sinusoidal endothelial cells of normal or PSC specimens. However, intense deposition of IgG and IgM on sinusoidal endothelial cells in the liver sections of PBC and AIH patients was observed. **c:** Double staining of PBC and AIH liver sections with anti-human IgG and IgM (CY-3, red) and anti-DC-SIGN2 (FITC, green) clearly showed coincident sites of reactivity (yellow) in many areas. Thicker and more intense deposition of immunoglobulins was observed in PBC livers as compared to AIH specimens.

fenestrae, eg, vascularization,³⁻⁵ and they lose some of their phenotypic features regardless of whether sinusoidal vascularization occurs.^{20,21} In addition, studies have shown endothelial cell damage in PBC.⁶ High serum levels of circulating von Willebrand factor antigen (an endothelial-specific product)⁶ and hyaluronic acid⁵ have been found in patients with cirrhosis, including PBC, and shown to correlate with impaired hepatic synthetic function. These findings therefore reflect injury and impaired endothelial cell function in PBC patients. Our results show that Abs to LSECs may, in part, play an important role in this damage or decreased endothelial cell function. Abs to LSECs may initiate significant alterations in LSEC structure and function. Thus, these Abs may contribute to the continuous production of basement membrane proteins by the phenotypically altered endothelial cells in the fibrotic liver. The thicker and more intense *in vivo* deposition of IgG and IgM immunoglobulins in and around the sinusoidal endothelial cells in PBC liver sections further support our finding regarding the role of these antibodies in causing clinically relevant changes in the livers of these patients.

Interestingly, during organogenesis, the liver anlage is vascularized by continuous capillaries with basement membrane, but between 5 and 20 weeks of gestation the vessels in the immediate proximity of hepatocytes become fenestrated, lacking specialized junctions and basement membrane, suggesting the production of signals capable of modulating the endothelial phenotype.²² These events are reversed during cirrhosis such that there is transformation from a sinusoid to a vascular phenotype. The signals involved in these transformation processes either during organogenesis or cirrhosis are currently not known. Normal sinusoidal endothelial cells express mRNA for CD31, intracellularly express the protein, but no cell surface expression is observed. However, anti-LSEC Abs induce the cell surface expression of this protein. Thus, anti-LSEC Abs now provide a means for studying the transformation-inducing signals produced during cirrhosis. Likewise, this knowledge may help shed some light on the molecular events involved in transformation of a vascular endothelium into a sinusoid during organogenesis.

Our efforts to assess reversibility of transformed LSECs back to a sinusoid, following removal of Abs gave interesting results. Although the experiments were standardized with regard to the amount and titers of antibodies used, AIH and PBC sera gave different results. Removal of AIH anti-LSEC Abs *in vitro* completely reverted transformed LSECs (within 4 weeks) back to a sinusoid, while removal of PBC anti-LSEC Abs did not (even after 3 months). We speculate that differences in the specificities or modes of action of the Abs binding to LSECs in AIH and PBC may vary and could be responsible for this observation. Identification of the antigen(s) recognized by these Abs will not only elucidate important molecules involved in vascularization but may provide a better understanding of cirrhosis and even suggest therapeutic approaches. Such studies are currently in progress using the technology of proteomics and DNA microarray assays.

It is important to keep in mind that the detection of anti-LSEC Abs was performed on serum samples taken at only one time point. Monitoring of the patients at different time points will be important to determine the true frequency of these Abs in ALD patients. Thus, it may be likely that patients without detectable anti-LSEC Abs in this study may have these Abs at other time points. A study to detect the presence of anti-LSEC Abs at different time points in these patients has been initiated.

Our results indicated that anti-LSEC Abs not only induced transformation of LSECs but also induced the production of the chemokines IL-8 and MCP-1. IL-8 is a potent chemotactic factor for neutrophils, lymphocytes, and eosinophils.^{23,24} Significantly increased numbers of eosinophils have been reported in PBC livers.^{25,26} Our results indicate that anti-LSEC Abs may be one factor responsible for the increased numbers of eosinophils via production of IL-8. MCP-1 belongs to the C-C class of chemokines and binds to the CCR2 chemokine receptor.²⁷ Several cell types, including basophils, monocytes, activated T cells, dendritic cells, and natural killer cells²⁷ express the CCR2 receptor, allowing MCP-1 mediated biological effects. The major role of MCP-1 appears to be in recruitment of mononuclear cells to sites of inflammation. Thus both IL-8 and MCP-1 production by LSECs induced by Abs to LSECs will contribute to recruitment of monocytes and lymphocytes into the inflamed liver in PBC and AIH. Leukocyte-endothelial interactions can signal further release of cytokines and chemokines, which exacerbates the inflammatory process resulting in chronic inflammation.

Both IL-6 and TGF β -1 were found to be constitutively produced by liver sinusoidal endothelial cells, the production of which was decreased after treatment with anti-LSEC antibodies. In normal rat liver TGF β -1 levels have been found to be relatively high in sinusoidal endothelial and Kupffer cells.²⁸ However, it has been shown that in a fibrotic injury model, TGF β mRNA was increased only in stellate cells, but not endothelial cells.²⁸ It is therefore likely that even though normal LSECs secrete TGF β -1 during fibrosis or after injury, TGF β -1 increases selectively in the stellate cells, while LSECs either produce decreased levels or none of this cytokine. It is unlikely that TGF β is the factor inducing basement membrane production, since this cytokine was also produced by normal LSECs.

The expression of CD31, VCAM-1, and increased expression of ICAM-1 on transformed sinusoidal cells by anti-LSEC Abs during cirrhosis reflects the fact that the expression of these adhesion molecules can now facilitate leukocyte adhesion to, and diapedes across, the endothelium.²⁹

In terms of basement membrane formation, the first detectable response of the liver to chronic injury is increased fibronectin deposition in the space of Disse.³⁰ Some time after fibronectin deposition is obvious, increased deposition of collagen type I followed by collagen type IV and later by laminin occurs.³¹ The cellular origin of the ECM deposited during the cirrhotic process has been the subject of some controversy. The stellate cell is believed to be the main contributor^{32,33}; however,

hepatocytes and fibroblasts have also reported to contribute to ECM deposition.^{34–36} In this study we show that sinusoidal endothelial cells secrete collagen type IV and laminin after activation with anti-LSEC autoantibodies. Both endothelial and stellate cells have been shown to secrete these basement membrane components³⁰; however, the extent of their relative contribution is still not established.

So far, most of the autoantibodies described in the sera of ALD patients are directed to intracellular antigens and do not correlate to any clinical parameter. Therefore, in the past, the importance and contribution of autoantibodies in inflammatory processes involved in autoimmune liver diseases has been underestimated. However, we show that Abs reactive with the clinically relevant target cells of immune attack in these patients may have an important role to play in the pathogenesis and progression of these diseases. The present study describes for the first time the clinical importance of autoantibodies to cell surface antigens expressed on liver sinusoidal endothelial cells. We demonstrate clearly the transformation of sinusoidal endothelial cells to a vascular type by these Abs. The specificity of the Abs is different from AMA, ANA, and antibodies reacting with HUVECs and HKMECs. The functional capacity of the Abs to transform the LSECs appears to reside only in the fraction that is reactive with LSECs. This is demonstrated by the fact that absorption with LSECs completely removed the capacity of the F(ab)₂ Ig fractions to induce transformation of LSECs. However, reactivity to mitochondria, nuclear antigens, HUVECs, and HKMECs was still maintained even after absorption. Thus, in PBC and AIH patients these Abs may be directed to LSEC-restricted antigen(s).

In conclusion, our findings describe the presence of autoantibodies reactive with probably novel cell surface antigens expressed on liver sinusoidal cells that are clinically important in the pathogenesis and progression of PBC and AIH. Our results suggest that anti-LSEC Abs may play an important role in the development of hepatocellular failure and portal hypertension in PBC and AIH patients by causing endothelial cell transformation. Identification of the antigen(s) recognized by these autoantibodies will not only elucidate important molecules involved in vascularization but also may provide a better understanding of cirrhosis and even suggest therapeutic approaches. Our results have other important biological implications. Using anti-LSEC Abs as a means to study the transformation signaling processes in the sinusoid during development of cirrhosis may help understand some of the factors responsible for transformation of vascular endothelium into a sinusoid during organogenesis.

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